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ORIGINAL ARTICLE

Viral load and clinical features in children infected with seasonal influenza B in 2006/2007†

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Background/Purpose: In influenza B infection, viral load is believed to be related to the severity of clinical illness. The correlation between viral load and symptoms is not known. We conducted a study to assess the relationship between virus load and clinical features in children infected with influenza B, in the hope that clinical features could be used as surrogate markers of viral load to guide treatment.
Methods: Between December 2006 and February 2007, 228 patients with fever and respiratory symptoms were prospectively enrolled in our tertiary hospital-based study. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed to determine viral load.
Results: Real-time RT-PCR was positive for influenza B in 76 patients. Using virus culture as the gold standard, the sensitivity and specificity were 95% and 87%, respectively. Influenza culture positive rate significantly correlated with viral load ($p = 0.03$). The median copy number of influenza B virus in the 76 RT-PCR positive patients was 9735 copies/ml (range 4.8×10^{1}–2.0×10^{6} copies/ml). Samples obtained later in the clinical course tended to have lower viral load

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($p = 0.7$), while patient age ($p = 0.72$) and fever duration ($p = 0.96$) positively related to viral load. In patients >3 years of age, myalgia was related to statistically lower viral loads (14300 vs. 1180; $p = 0.025$). Patients with chills tended to have higher viral loads (72450 vs. 7640; $p = 0.1$). Patients with abdominal pain tended to have lower viral loads (1998 vs. 12550; $p = 0.06$).
Conclusion: Culture rate positively correlated with viral load. Patients with myalgia had a lower viral load.

Introduction

Influenza is a common disease, which is associated with outpatient visits, hospitalizations, morbidity, and mortality in children.[1] It has a short incubation period and is easily transmitted. Effective medication, rapid diagnosis, and timely intervention are of vital importance to the public.[2] Since the development of polymerase chain reaction (PCR) analysis in 1985, the sensitivity and speed of diagnosing various virus infections, including influenza, has been much improved.[2]

Real-time PCR, which allows the assessment of the amount of virus at a given point in time, has been used extensively to determine the dynamics of diseases caused by viral infections such as hepatitis B and human immunodeficiency virus (HIV).[3,4] Studies investigating the relationship between viral load and the disease course of natural human influenza infection have been limited to immunocompromised patients, H5N1 avian influenza, and pandemic H1N1.[5–8] This present study collected data regarding influenza B viral load, during natural infection in children, and investigated how viral load correlates with clinical manifestations of the disease.

Materials and methods

In the peak of the 2006/2007 influenza season, between December 8, 2006 and February 8, 2007, we enrolled 228 patients <18 years old with influenza-like illnesses (ILI) who visited either the pediatric outpatient clinic, emergency departments, or were admitted to National Taiwan University Hospital, a tertiary hospital in Taiwan. ILI was defined as the presence of fever (tympanic temperature ≥38°C), plus any respiratory symptoms or signs. All patients or their parents gave written informed consent. Two throat swabs were taken from each patient: one for real-time reverse-transcriptase-PCR (RT-PCR) in our research lab, and one for virus culture in the central lab. For patients with positive results for influenza B virus (isolated virus or positive real-time RT-PCR), we analyzed their clinical manifestations, contact and vaccine history, diagnosis, and treatment by a review of their medical records. Pneumonia was diagnosed by positive radiological findings or notes recorded on patient charts. Sinusitis was diagnosed by clinical manifestations of purulent rhinorrhea, sinus tenderness, and/or haziness of the sinuses on sinus X-ray. Acute otitis media (AOM) was diagnosed by the presence of congested ear drums with or without effusion.

For virus isolation, throat swab samples were inoculated into human embryonic fibroblast (MRC-5), LLC-MK2, HEp-2, and RD cell cultures. Once cytopathic effects appeared, fluorescent antibody staining was used to confirm the presence of the influenza B virus. For real-time RT-PCR, RNA extraction from the throat swabs was performed using a viral RNA extraction kit in accordance with the manufacturer's instructions (QIAamp Viral RNA Mini Kit, Qiagen, Hilden, Germany). Light cycle real-time RT-PCR was used to detect influenza B viruses. Primer and probe sequences based on the HA gene, 5′-AAATACggTggATTAAATAAAAgCAA-3′, 5′-CCAgCAATAgCTCCgAAgAAA-3′, and the probe 5′-6FAM-CACCCATATTgggCAATTTCCTATggC −TMR-3′, were used. RT-PCR was performed at 61°C for 20 minutes, denaturing at 95°C for 30 seconds, 50 cycles of 95°C for 1 second, 56°C for 15 seconds, 72°C for 15 seconds, and cooling at 40°C for 30 seconds. Any copy number detected was considered a positive PCR result.

Chi-square or Fisher's exact test was used to analyze categorical data. Mann-Whitney *U* test was used for continuous variables not normally distributed. Linear regression was used for correlation analysis. All *p* values were two-tailed, and *p* values less than 0.05 were considered to be statistically significant. SPSS 14.0 (SPSS, Inc., Chicago IL) was used for statistical analysis.

Results

Sensitivity and specificity of influenza PCR

Real-time RT-PCR was positive for influenza B in 76/228 patients. Fifty-six were both RT-PCR and culture positive. Three culture-proven influenza B patients had negative RT-PCR results. Twenty patients were RT-PCR positive/culture negative. Among them, two were found to be culture positive for herpes simplex virus type 1 (HSV-1), one for cytomegalovirus (CMV), and one for parainfluenza virus type 3. No viruses could be isolated in the remaining influenza B RT-PCR positive/culture negative patients. Seventeen patients tested positive for influenza A by RT-PCR and culture ($n = 15$), or culture only ($n = 2$). Among those negative for both RT-PCR and culture, 34 specimens were culture positive for other pathogens including respiratory syncytial virus ($n = 8$), adenovirus ($n = 11$), parainfluenza virus ($n = 5$), non-polio enterovirus ($n = 5$), CMV ($n = 3$), and HSV-1 ($n = 2$).

Using viral isolation as the gold standard, any copy number found by real-time RT-PCR had a 95% sensitivity and 87% specificity in detecting influenza B. Influenza culture positive rate significantly and positively correlated with viral load (Fig. 1; $p = 0.03$). The specificity would be increased to 89% if a cut-off viral load of 10^2 is used,

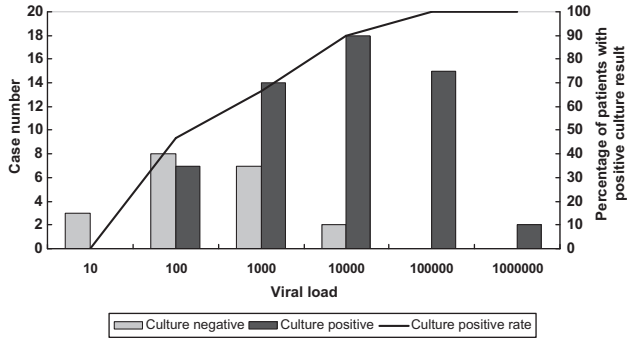

Figure 1 Virus culture result and its relationship with viral load in throat swab specimens from children infected with influenza B ($N = 76$). Viral load is positively correlated with culture positive rate ($p = 0.03$ by linear regression analysis).

whereas the sensitivity remained unchanged. The specificity would further increase to 94% where a cut-off viral load of 10^3 is used, but here the sensitivity would be decreased to 83%.

Demographic data and clinical manifestations of patients

We further analyzed the demographic and clinical data of 79 patients with influenza B, diagnosed either by culture or RT-PCR. Their median age was 7 years (range 9 months—17 years 11 months). Patients aged <5 years, between 5 years and 10 years, and >10 years, accounted for 33%, 42%, and 25% of all patients, respectively. Forty-eight (61%) patients were male.

Thirty-two percent of influenza positive patients (25/79) had contacted at least one symptomatic household member before symptom onset. Among them, 18 (72%) had family members with upper respiratory tract symptoms. Seven (28%), had family members with ILI. Most of the household contacts were brothers or sisters (19/25). Others were parents ($n = 3$) or grandparents ($n = 3$).

The most common initial diagnoses of these patients were influenza or ILI (53%) and pneumonia (18%). The most common signs and symptoms were rhinorrhea (77%), cough (71%), and throat injection (60%). Forty-one patients had their fever duration recorded and the median was 4 days (range 1—9 days).

Vaccination history

Ten (13%) patients had received at least one dose of influenza vaccine in the 2006/2007 season and all were younger than 8 years old. Three patients had received a complete two-dose vaccination course. Patients who were vaccinated were significantly younger than those who were not (median age = 30 vs. 98 months; Mann-Whitney *U* test, $p = 0.001$).

Disposition, treatment, and complications

Twenty percent of all patients (16/79) were hospitalized for a median of 6.5 days (range 1—20 days). Two patients had been admitted to the intensive care unit because of severe pneumonia and empyema. Oseltamivir (Tamiflu) had been given to seven patients. The duration of treatment ranged between 4 days and 7 days.

The most common complication was pneumonia (24%). Twenty-four patients underwent chest X-ray examination. Imaging studies showed lung consolidation in four, including one with a group A β-hemolytic streptococcal empyema who had undergone video-assisted thoracoscopic surgery. Other complications included sinusitis (20%) and AOM (10%). In multivariate analysis, consolidation on chest X-ray was the only factor that significantly affected the decision to admit a patient ($p < 0.01$).

Viral load

Defining the day of fever onset as day 1, the median sample time was day 3 (range 1—11). Fifty-six percent (44/79) of the samples were obtained within 3 days of fever onset. The median copy number of influenza B virus in the 76 RT-PCR positive patients was 9,735 copies/ml (range 48—2×10^6 copies/ml). There was a trend to suggest that sample time negatively correlated to viral load (Fig. 2; $r^2 = 0.002$; $p = 0.7$). Patient age (correlation coefficient = 0.041; $p = 0.72$) and fever duration (correlation coefficient = 0.006; $p = 0.96$) positively correlated with viral load.

Apart for patients with myalgia who had lower viral loads (median values = 2,440 vs. 13,000; $p = 0.04$), viral load did not differ between patients with different clinical manifestations and demographics (Table 1). Because myalgia is a subjective complaint that is more common in older children (median age = 110 vs. 83 months; $p = 0.04$ [Mann-Whitney *U* test]), we further stratified patients with myalgia into different age groups (≤3 years and >3 years) and compared their viral loads. There were 14 and 62 patients in each age group, respectively. Only in patients >3 years old did myalgia relate to a statistically lower viral load (14,300 vs. 1,180; $p = 0.03$ by Mann-Whitney *U* test). For patients >3 years old, we further stratified them by sample day. Among all samples taken ≤3 days after fever onset, myalgia was significantly associated with lower viral loads ($p = 0.047$ by Mann-Whitney *U* test). Patients with chills tended to have higher viral loads (72,450 vs. 7,640),

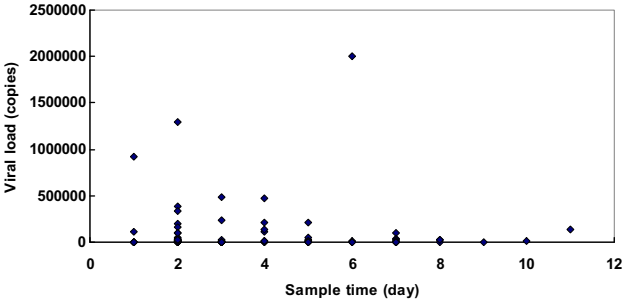

Figure 2 Correlation of viral load with sample time in children infected with influenza B ($n = 76$). The day of fever onset was defined as day 1. Correlation of viral load in throat swab specimen with sample time after fever onset ($r^2 = 0.002$; $p = 0.7$).

Table 1 Median viral load (VL_M) according to clinical features ($N = 76$).

Variable	VL_M with this feature (case number)	VL_M without this feature (case number)	*p* value
Male	9325 (46)	13,050 (30)	0.20
Influenza vaccine	12,550 (10)	9325 (66)	0.81
Admission	9900 (15)	9570 (61)	0.60
Complications	14,950 (36)	8480 (40)	0.76
Use of oseltamivir	9080 (7)	9900 (69)	0.96
Household contact	9080 (25)	9900 (51)	0.64
Rhinorrhea	9570 (57)	10,500 (19)	0.90
Cough	8480 (66)	17,100 (10)	0.25
Sore throat	19,050 (16)	8480 (60)	0.38
Hoarseness	7200 (3)	9900 (73)	0.93
Calf pain	8840 (18)	12,100 (56)	0.60
Myalgia	2400 (14)	13,000 (62)	0.04*
Chills	72,450 (10)	7640 (66)	0.10
Headache	15,300 (11)	9570 (65)	0.87
Abdominal pain	1998 (8)	12,550 (68)	0.06
Vomiting	15,300 (7)	9080 (69)	0.87
Diarrhea	16,600 (10)	9325 (66)	0.87

*Statistically significant by Mann-Whitney *U* test.

whereas patients with abdominal pain tended to have lower viral loads (1,998 vs. 12,550), but these differences did not reach statistical significance.

Discussion

The sensitivity and specificity of influenza PCR varies in different studies, depending on the technique, patient population, and gold standard used.[9] The sensitivity and specificity of our influenza RT-PCR is similar to that reported in other series.[10] Detection methods using conventional cultures have always had some disadvantages due to the lack of speed and the little impact on patient care. In previous studies, culture positive rate was found to be associated with semi-quantitative results of RT-PCR when comparing the cycle threshold value.[9,11] Higher viral load was consistently found in culture-positive samples, although the statistical significance had never been proved. Our study clearly demonstrates the correlation between viral load and a positive viral culture. In our study, samples with copies of viral RNA fewer than 1000 would not yield a positive culture result in more than half of cases. Because the presence of viral genome does not necessarily mean that there are replication-competent viruses in the clinical samples, this discrepancy is understandable. We believe that positive RT-PCR results in patients with clinical symptoms of influenza indicate active infection.

In our study, real-time RT-PCR detected influenza in 56 of 59 culture-positive samples, and resulted in 20 additional positives. Because all of these patients had fever and respiratory symptoms, these 20 cases are unlikely to be false positives. Five of these patients were also culture positive for other pathogens. Because no typical symptoms of CMV or HSV infection were recorded in these patients, the presence of CMV and HSV may reflect colonization rather than true infection. The patient who was RT-PCR positive for influenza B and culture positive for parainfluenza virus type 3 may had dual infection, which is not a rare condition.[12] Three patients were RT-PCR negative, but culture positive. In a previous study comparing RT-PCR with conventional culture, viral genome was detected by RT-PCR in eight out of nine culture-positive specimens.[9] Because no single test is 100% sensitive and specific, based on the results of our study, and also clinical experience, we believe that RT-PCR complements, rather than replaces, conventional culture as a diagnostic tool.

The low hospitalization rate (around 20%) reflects the outpatient source of our patients. Apart from younger age,[13] radiographic findings of pulmonary consolidation is also a risk factor for hospitalization. Pulmonary complications can be caused by influenza virus itself, by secondary bacterial infection, or both.[13] *Streptococcus pneumoniae* and *Staphylococcus aureus* are the bacterial pathogens most commonly identified.[14–16] Group A β-hemolytic streptococcus has also been reported.[15] As we had only one patient who had undergone invasive diagnostic procedures, the bacterial etiology remained unknown in most subjects.

The viral kinetics of influenza virus A infection within an individual has been previously studied using experimental infections.[17,18] There is also a significant correlation between viral titers and cytokine levels, which reportedly peaks at different days after infection.[18] In natural infections, the virus load can be affected by different inoculation routes and amount, as well as patient age, and their general health condition. In a recent study on viral load in patients infected with pandemic H1N1 influenza A published by To et al, serial samples were taken from patients hospitalized for pandemic H1N1, and viral load was found to be negatively correlated with time after symptom onset.[8] The correlation between virus load and days after infection was not as clearly identified in our study because we did not have serial samples from the same patient. Serial viral load monitored in each patient with simultaneous cytokine assays will help us understand more about the viral kinetics of natural infection and the host response. Lack of serial sampling is the main limitation of our study, but our setting is more similar to the situation found in daily practice.

In order to find any clinical parameter that could predict a higher viral load and help in clinical decision-making, we attempted to find the correlation between viral load and various clinical manifestations. In patients older than 3 years, in whom we assume that subjective complaints are more reliable, complaints of myalgia were associated with a lower viral load, irrespective of sample day. In a study focusing on the relationship between cytokine responses, viral load and clinical symptoms of experimental human influenza A virus infection in 20 adult volunteers, viral load and systemic symptoms, such as fever and muscle ache, both peaked at the second day of infection.[18] Serum level of interleukin (IL) peaked on the second day, whereas tumor necrosis factor (TNF) peaked at the 4th day, when the severity of systemic symptoms also decreased.[18] In the above study, the severity score of all systemic symptoms (muscle aches, fatigue, headache, and fever) were combined as a generalized indicator of the severity, which

made the assessment of individual symptoms unfeasible. As myalgia was purported to be caused by TNF and prostaglandin,[19] it is reasonable to assume that the presence of myalgia is associated with higher level of TNF and a lower viral load. The trend of chills associating with a higher viral load can also be explained by the effect of IL on fever/chills.[19]

In summary, this study defines the correlation between viral load and culture positivity. We also suggest that myalgia is an indicator of a lower viral load in patients. The usefulness of clinical manifestations as surrogate markers of infection severity to guide clinical treatment is worthy of further investigation.

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